Gut and immune effects of bioactive milk factors in preterm pigs exposed to prenatal inflammation

Ren, Shuqiang; Hui, Yan; Goericke-Pesch, Sandra; Pankratova, Stanislava; Kot, Witold; Pan, Xiaoyu; Thymann, Thomas; Sangild, Per T; Nguyen, Duc Ninh

Published in:
American Journal of Physiology: Gastrointestinal and Liver Physiology

DOI:
10.1152/ajpgi.00042.2019

Publication date:
2019

Document version
Peer reviewed version

Gut and immune effects of bioactive milk factors in preterm pigs exposed to prenatal inflammation

Shuqiang Ren[1], Yan Hui[2], Sandra Goericke-Pesch[3], Stanislava Pankratova[1], Witold Kot[4], Xiaoyu Pan[1], Thomas Thymann[1], Per T. Sangild[1,5,6], Duc Ninh Nguyen[1*]

[1]*Section for Comparative Pediatrics and Nutrition, Department of Veterinary and Animal Sciences, University of Copenhagen, Denmark*

[2]*Department of Food Science, University of Copenhagen, Denmark*

[3] *Department of Veterinary Clinical Sciences, University of Copenhagen, Denmark*

[4] *Department of Environmental Sciences, Aarhus University, Denmark*

[5] *Department of Pediatrics and Adolescent Medicine, Rigshospitalet, Copenhagen, Denmark*

[6] *Department of Pediatrics, Odense University Hospital, Odense, Denmark*

Running title: Bioactive milk diets in preterm neonates born with prenatal inflammation

*Corresponding author: Duc Ninh Nguyen, Section for Comparative Pediatrics and Nutrition, Department of Veterinary and Animal Sciences, University of Copenhagen, Dyrlægevej 68, DK-1870 Frederiksberg C, Denmark. Email: dnn@sund.ku.dk

Key words: bovine colostrum, caseinoglycomacropeptide, lipopolyssacharide, osteopontin, prenatal inflammation, preterm pigs

Abstract

Prenatal inflammation may predispose to preterm birth and postnatal inflammatory disorders, such as necrotizing enterocolitis (NEC). Bioactive milk ingredients may help to support gut maturation in such neonates, but mother´s milk is often insufficient after preterm birth. We hypothesized that supplementation with bioactive ingredients from bovine milk (osteopontin, OPN; caseinoglycomacropeptide, CGMP; colostrum, COL) supports gut, immunity and NEC resistance in neonates born preterm after gram-negative infection before birth. Using preterm pigs as a model for preterm infants, fetal pigs were given intra-amniotic injections of lipopolysaccharide (LPS, 1 mg/fetus) and delivered three days later (90% gestation). For five days, groups of LPS-exposed pigs were fed formula (FOR), bovine colostrum (COL), or formula enriched with OPN or CGMP. LPS induced intra-amniotic inflammation, postnatal systemic inflammation but limited effects on postnatal gut parameters and NEC. Relative to FOR, COL feeding to LPS-exposed pigs showed less diarrhea, NEC severity, reduced gut IL-1β and IL-8 levels, greater gut goblet cell density and digestive enzyme activities, and blood helper T cell fraction. CGMP improved neonatal arousal, gut lactase activities and reduced LPS-induced IL-8 secretion in intestinal epithelial cells (IECs) *in vitro*. Finally, OPN tended to reduce diarrhea and stimulated IEC proliferation *in vitro*. No effects on villus morphology, circulating cytokines or colonic microbiota were observed among groups. In conclusion, bioactive milk ingredients exerted only modest effects on gut and systemic immune parameters in preterm pigs exposed to prenatal inflammation. Short-term, prenatal exposure to inflammation may render the gut less sensitive to immune-modulatory milk effects.

New and noteworthy: Prenatal inflammation is a risk factor for preterm birth and postnatal complications including infections. However from clinical studies, it is difficult to separate the effects of only prenatal inflammation from preterm birth. Using caesarean-delivered preterm pigs with prenatal inflammation, we documented some beneficial gut effects of bioactive milk diets, relative to formula, but prenatal inflammation appeared to decrease the sensitivity of enteral feeding. Special treatments and diets may be required for this neonatal population.

Introduction

Preterm births (<37 weeks of gestation) represents 15 million infants every year (10% of all pregnancies), and is the most important cause of neonatal mortality (5, 6). Prenatal infection and inflammation, including chorioamnionitis (CA), inflammation of the fetal membranes, is a main predisposing factor for preterm birth (40-70% cases) (14, 16, 25). The incidence of CA is inversely related to gestational age at birth, and many CA cases are related to infection with the low-virulent bacteria, Ureasplasma (15, 59). CA may be associated with increased risks of neonatal early-onset sepsis (EOS) (2, 24), necrotizing enterocolitis (NEC) (1, 3), and neurodevelopmental disorders (58), but it remains elusive if these effects are direct or indirect. In addition, it is unclear how the first milk diets (mother´s own milk, standard formula, enriched formula) (11) may modify the gut and systemic responses to prenatal inflammation. For preterm infants with CA, sub-optimal neonatal feeding may induce a second inflammatory insult to further increase the risk of infection and NEC (53). Our recent study on formula-fed preterm pigs has shown that CA induced by 3 days of intra-amniotic (IA) exposure to LPS exerted strong fetal gut responses and postnatal systemic inflammation but did not increase NEC sensitivity on day 5 (33). Thus, CA may predispose to EOS in preterm infants while the effects on NEC are more unclear. Sheep studies have shown CA-induced fetal inflammatory responses in the gut, lung and brain at birth (26, 50), but neither of these earlier studies allowed postnatal recovery and recording of inflammation and NEC sensitivity after enteral feeding. Possibly, an optimized milk diet for preterm neonates may not only protect the immature gut against NEC, but also reverse negative impacts of CA on the postnatal systemic immunity and inflammation in multiple organ systems.

In preterm pigs delivered by caesarean section after unaffected pregnancies, feeding bovine colostrum (COL) reduces NEC incidence, improves gut maturation, and down-regulates expression of intestinal genes related to inflammation, relative to infant formula (20, 37, 48, 49). Initial preterm infant studies also show that COL decreases diarrhea (51) and the time to full enteral feeding (22). Similar to

mother's milk, COL contains multiple bioactive ingredients including anti-bacterial, anti-inflammatory proteins and peptides (immunoglobulins, lactoferrin, transforming growth factor β, casein, osteopontin (OPN)) (10). Bioactive components in COL may provide a better option or supplement than conventional infant formula for preterm infants, and may help to reverse gut inflammation induced by prenatal inflammation in infants born after CA. Among the milk bioactive components, OPN is an important protein with higher levels in COL than in bovine mature milk and human milk (29). It exerts anti-inflammatory properties (38) and anti-bacterial activities as well as protects the epithelial barriers via interactions with macrophages and neutrophils (23, 54, 60). OPN-enriched formula fed to newborn infants shows systemic immune-modulatory effects with lower plasma levels of TNF-α and a higher frequency of blood T cells than regular infant formula (29, 55). Caseinoglycomacropeptide (CGMP) is another promising milk peptide derived from kappa-casein with multiple immune-modulatory effects. For instance, CGMP decreases inflammatory gene expressions in porcine intestinal epithelial cells *in vitro* (18), downregulates the secretion of pro-inflammatory cytokines IFN-γ and TNF-α on rat splenocytes (41). CGMP is also considered a prebiotic component, via its decoy functions against pathogenic *E.coli* adhesion to the gut mucosa (8, 17), and via its ability to promote the growth of *Bifidobacteria* and *Lactococcus* spp. (7, 42).

Based on the already reported immune-modulatory effects of COL, OPN and CGMP, and the effects of prenatal inflammation on postnatal gut and systemic inflammation (33), we hypothesized that feeding COL, or formula (FOR) enriched with OPN or CGMP, would be superior to FOR feeding alone in protecting against NEC and systemic inflammation in preterm neonates born after prenatal inflammation. Preterm pigs were given an IA dose of LPS, as previously described (33), and were fed one of the above four diets for five days. The study primary outcome was NEC incidence and secondary outcomes included gut functions (absorption, enzyme activities, permeability), gut inflammation (inflammatory cytokines), and systemic immune endpoints (hematology and neutrophil functions).

Material and Method

Preterm pig experiments

All animal procedures were approved by the Danish National Committee of Animal Experimentation licence number 2014−15−0201−00418, which is in line with directive 2010/63/EU from the European Parliament. The schematic overview of the animal experimental design was depicted in Fig. 1. Eight pregnant sows (Large White x Danish Landrace x Duroc) were operated by laparotomy at d 103 of gestation (term at 117±2 days of gestation), and each fetus received either an IA dose of 1 mg LPS (LPS, n = 141, from *E.coli* 055:B5, Sigma Aldrich, Copenhagen, Denmark) or control (CON, n = 47, saline or no injection) in an area close to the mouth, as previously described (33). Post-surgical monitoring of the sows included frequent clinical evaluation and temperature measurement until the time of planned delivery to record any potential signs abortion or infection. Preterm pigs were then delivered by caesarean section at d 106 (89-92% gestation age). In experiment 1, to confirm the postnatal gut and systemic effects of prenatal LPS, a proportion of the surviving pigs at birth with and without IA LPS injection (25 CON and 26 LPS pigs) were fed formula until euthanasia on postnatal d 5. In experiment 2, to test the effects of bioactive milk components in pigs born with prenatal inflammation, 44 caesarean delivered piglets with IA LPS injection from five of the above eight pregnant sows were block-randomized, according to birth weight and gender, into four groups fed with four different diets: formula (FOR, n=11), bovine colostrum (COL, n=10), casein-glycomacropeptide-enriched formula (CGMP, n=11) and osteopontin-enriched formula (OPN, n=12). Pigs that died before birth or during the first 24 h after birth were excluded from the data analyses.

After birth, each piglet was transferred to a pre-heated individual incubator (37-38^{o}C) with supplemental oxygen (0.5-2 L/min, for the first 24 h). If necessary, resuscitation was performed by physical stimulation and positive airway ventilation until stable respiration. Each pig was then inserted a vascular catheter (4F, Portex, Kent, UK) via the umbilical artery for blood sampling and parenteral nutrition (PN) and an orogastric catheter (6F, Portex, Kent, UK) for enteral nutrition (EN), as described previously (21). During

the first 24 h, piglets also received maternal plasma (16 mL/kg) via the umbilical catheter for provision of passive immunity. All piglets were nourished by PN (Kabiven, 3210 kJ/L, modified to meet piglet nutrient requirement (44, 47), gradually decreased amount, 96-48 mL/kg/d), and by EN (gradually increased amount of 24-120 mL/kg/d) until postnatal d 5, when they were euthanized for tissue collection. Four enteral diets were designed isoenergetically (Table 1). Infant formula consisted of whey protein concentrate Lacprodan 80D (Arla Food Ingredients, Viby, Denmark), Seravit-SHS, Liquidgen MCT-SHS, Calogen LCT and Fantomalt (Nutricia, Birkerød, Denmark). For enriched formulas, the levels of supplemented CGMP and OPN were 30 g/L and 2.2 g/L, respectively. Bovine colostrum powder (Colodan, Biofiber Damino, Gesten, Denmark) was a commercial product previously used in clinical trials of preterm infants (22). Clinical conditions and faecal characteristics were evaluated twice daily as previously described (33). The time from birth until first signs of eyelid opening, standing and walking were recorded. At euthanasia, organs were weighed and tissues were snap-frozen and stored at -80^{o}C or fixed in paraformaldehyde 4% for later analyses. Five regions of the gastrointestinal tract including stomach, proximal, middle and distal small intestines and colon were evaluated for macroscopic NEC-like lesions by a scoring system from 1 to 6, as previously described (21). A pig with a score of at least 3 in any the five gastrointestinal regions was defined as NEC.

Hematology and systemic immune analyses

At birth, amniotic fluid was collected for manual leukocyte counting to evaluate the levels of intra-amniotic inflammation, as previously described (33). Arterial blood (d 1, 3 and 5) was collected for hematology by an automatic cell counter (Advia 2120i Hematology System Siemens, Germany) and systemic immune analyses including blood T cell phenotyping and blood neutrophil phagocytosis function. For blood T cell characterization, blood erythrocytes were lyzed (1 × BD FACS Lysing solution, BD Biosciences, Lyngby, Denmark) and the remaining leukocytes were permeabilized (Fixation/Permeabilization Concentrate, eBioscience, ThermoFisher, Roskilde, Denmark) for 30 min at 4^{o}C in the dark and washed with permeabilization buffer (eBioscience). The cells were incubated 15 min in the

dark at 4^{o}C with porcine serum (Thermofisher) for Fc receptor blocking, and then stained with a mixture of PerCP-Cy5.5 conjugated anti-pig CD3 antibody (IgG2a isotype, BD Biosciences, Lyngby, Denmark), FITC-conjugated anti-pig CD4 antibody (IgG2b isotype, BioRad, Copenhagen, Denmark), PE-conjugated mouse anti-pig CD8 antibody (IgG2a isotype, Biorad) and APC-conjugated anti-mouse/rat Foxp3 antibody (IgG2a isotype, eBioscience). Samples were then analyzed by BD Accuri C6 flow cytometer (BD Biosciences). PerCP-Cy5.5-conjugated mouse IgG2a negative control antibody (BD Bioscience), APC-conjugated rat IgG2a negative control antibody (eBioscience), PE-conjugated mouse IgG2a negative control antibody and FITC-conjugated mouse IgG2b negative control antibody were used as isotype controls. T cell subsets were analyzed including helper T cells (Th, $CD3^{+}CD4^{+}CD8^{-}$ lymphocytes), cytotoxic T cells (Tc, $CD3^{+}CD4^{-}CD8^{+}$ lymphocytes) and regulatory T cells (Treg, $CD3^{+}CD4^{+}Foxp3^{+}$ lymphocytes). Blood neutrophil phagocytosis function was analyzed by flow cytometry using the pHrodo Red E.coli (560/585 nm) Bioparticles Phagocytosis Kit for Flow cytometry (Thermofisher) as previously described (32). Phagocytic rate was assessed as percentage of neutrophils exerting phagocytosis ($pHrodo^{+}$ neutrophils), and phagocytic capacity was assessed as the fluorescent intensity of $pHrodo^{+}$ in the $pHrodo^{+}$ neutrophil population.

Serum biochemistry, tissue cytokines and brush border enzyme activities

At euthanasia, serum samples were collected for biochemical analyses by Advia 1800 Chemistry System (Siemens, Germany). Distal small intestinal tissues were homogenized and analyzed for IL-1β, IL-8, IL-10 and I-FABP by ELISA (R&D Systems, Abingdon, UK). In addition, the small intestinal homogenates (proximal, middle and distal regions) were also analyzed for brush border enzyme activities including peptidases (aminopeptidase N (ApN), aminopeptidase A (ApA) and dipeptidylpeptidase IV (DPPIV)) and disaccharidases (sucrase, maltase and lactase) by spectrophotometry, as previously described (45, 46).

Gut histology and goblet cell density

Fixed tissues from the small intestinal regions (proximal, middle and distal) were embedded in paraffin, sectioned and stained with hematoxylin and eosin for measurement of villus height and crypt

depth by ImageJ (version 1.50i, NIH, USA). Mucin-containing goblet cells in the distal small intestine and colon were stained with Alcian blue and Periodic acid-Shiff (AB-PAS) and quantified, as previously described (28).

Gut microbiota composition

Colon contents collected at euthanasia were used to extract total cellular DNA using PowerSoil DNA Isolation Kit (MoBio Laboratories, CA, US) (27, 40). The bacterial V3 region of 16S rRNA was chosen for NextSeq paired-end 150bp amplicon analysis using primer compatible with Nextera Index Kit (Illumina, CA USA): NXt_388_F: 5'-TCGTCGGCAG CGTCAGATGT GTATAAGAGA CAGACWCCTA CGGGWGGCAG CAG-3' and NXt_518_R: 5'-GTCTCGTGGGC TCGGAGATGTG TATAAGAGAC AGATTACCGC GGCTGCTGG-3' (Integrated DNA Technologies; Leuven, Belgium).

***In vitro* cell culture, proliferation and cytokine secretion**

IPEC-J2, an intestinal epithelial cell line (IEC), isolated from jejunum of a neonatal, unsuckled piglet (ACC 701, DSMZ, Braunschweig, Germany) was cultured in Advanced Dulbecco's Modified Eagle Medium /Ham's F-12 (DMEM/F-12) supplemented with heat inactivated 5% fetal bovine serum, 1% GlutaMAX and 0.2% penicillin-streptomycin (all from Gibco, New York, USA), at 37 ℃ and 5% CO_2. All experiments were performed in 3-4 independent replicates at cell passages 5-15.

For proliferation assay, the cells were seeded at 5×10^4 cells/mL in a 96-well plate, cultured for 24 h, and then serum starved for another 24 h. Thereafter, the cells were stimulated with bovine OPN (Lacprodan OPN-10, Arla Foods Ingredients, Viby, Denmark) or CGMP (Lacprodan CGMP-20, Arla Foods Ingredients) at various doses of 0.01-1 g/L (sterile filtered at 0.22 um) for 24 h, and then incubated with the CellTiter 96 AQ_{ueous} One Solution Reagent (Promega, Nacka, Sweden) for 4 h at 37 ℃ and 5% CO_2 before measurement of the absorbance at 490 nm. Cell proliferation was then quantified relative to controls (set as 100 %), which was cultured in the same serum-free medium.

For cytokine secretion assays, the cells were seeded at 1×10^5 cells/ml in a 24-well plate, cultured until reaching 70-80% confluence, and serum starved for 24h. The cells were then stimulated with 0.05 g/L CGMP or OPN with or without LPS (1 μg/mL, 0127:B8; InvivoGen, Toulouse, France) for 24 h. Supernatants were collected for analysis of IL-8 (specific porcine ELISA DuoSet kits, R&D Systems, Abingdon, UK).

Statistical analysis

All statistical analyses were performed using R, version 3.4.3. *In vitro* data including cell proliferation and secreted cytokines were analysed by linear models with protein concentration and passage as fixed factors (proliferation) or with treatment (with or without CGMP/OPN treatment) and cell passage as fixed factors (cytokines), using lmer function. Incidences of diarrhea and NEC and first stand were compared using Fisher exact tests. NEC severity score was analysed by non-parametric Mann-Whitney test. All other continuous data from the animal experiment (except gut microbiota) were analysed by linear mixed models with diet as a fixed factor, and litter as a random factor using lmer function. The post hoc Dunnet tests were used to compare interventions with the negative control (FOR). Data transformation (log or sqrt) was performed if necessary. Residuals and fitted values were used to evaluate normal distribution and variance homogeneity. Data are shown as mean ± SEM, and P values < 0.05 were regarded as statistically significant and P values < 0.1 were considered as tendencies of being significant.

For microbiome analysis, the raw sequencing reads were merged and trimmed, and chimeric reads were removed, resulting in zero-radius Operational Taxonomic Units (zOTUs) with UNOISE implemented in Vsearch (12, 13, 43). The green genes (13.8) 16s rRNA gene database was used as reference for annotation. Qiime pipeline (v1.9.1) (9) together with R packages vegan, ggpubr and ggplot2 were used for data analysis. All the samples were rarefied to the minimum sampling depth (32719 counts) for alpha diversity index calculation. The raw OTU table was normalized with cumulative sum scaling (39) to calculate unweighted and weighted Unifrac distance, and adonis from R package (vegan)(36) was performed to test differences

among treatments after removing the litter effect. Specific taxa comparisons were analysed by ANCOM with FDR correction (30). P values < 0.05 were considered as statistically significant.

Results

Effects of fetal LPS exposure on postnatal gut outcomes and systemic inflammation in preterm pigs

In experiment 1 testing fetal and postnatal effects of IA LPS, the incidence of fetal death was higher in LPS vs. CON pigs (45/141 vs. 4/47, $P < 0.01$). Total leukocyte levels in the amniotic fluid were highly elevated in LPS vs. CON pigs ($P < 0.001$, Fig. 2A), indicating LPS-induced IA inflammation. LPS pigs took longer time to stand for the first time, approximately 49 h in LPS vs. 31 h in CON pigs ($P < 0.05$, Fig. 2B). At euthanasia on day 5, circulating parameters showed responses to LPS with a tendency to increased IL-1β and glucose levels ($P = 0.09$ and 0.07, respectively), increased iron level ($P < 0.01$) and decreased levels of albumin and alanine aminotransferase activities (ALT), relative to CON pigs ($P < 0.05$ and 0.01, respectively, Fig. 2C-G). In contrast, gut parameters on day 5 showed no clear LPS effect on the measured inflammatory endpoints or NEC incidence (35-48%, Fig. 2H). Lactase activity across three regions of the small intestine did not differ between LPS and CON pigs (Fig. 2I), but it showed tendency to be lower in the middle small intestine of LPS vs. CON pigs ($P = 0.07$).

Clinical evaluations and physical activity in LPS-exposed preterm pigs

There was a tendency for more CGMP pigs to be on their feet within the first two days, relative to the FOR group ($P = 0.06$, Fig. 3A). During the study, diarrhea occurrence was dominant in FOR pigs (91%), but lower in the remaining groups (40% in COL pigs, 4/10, $P < 0.05$; 55% in CGMP pigs, 6/11, $P = 0.15$; 58% in OPN pigs, 7/12, $P = 0.15$, Fig. 3B). NEC incidence in the whole gastrointestinal tract was not different among the groups but the NEC severity score tended to be lower in COL vs. FOR pigs ($P = 0.08$, Fig. 3C-D).

Intestinal morphology, brush border enzyme and nutrient absorption in LPS-exposed preterm pigs

Gut morphology data showed similar villus height (Fig. 4A) and crypt depth (Fig. 4B) across all the three regions of the small intestine. FOR pigs had the lowest lactase activities across three intestinal regions, relative to the other three groups (Fig. 4C). Comparing lactase activity in each specific small intestinal

region, COL pigs had 1.5 to 2-fold higher level than FOR pigs in proximal, middle and distal regions ($P < 0.01$, $P < 0.001$ and $P = 0.07$, respectively), and CGMP pigs had higher levels than FOR pigs only in middle region ($P < 0.01$, Fig. 4C). Plasma galactose levels following administration of a bolus of galactose solution, used as a hexose absorption marker, showed numerically higher values in CGMP vs. FOR pigs (Fig. 4D).

Gut cytokines, proteins and goblet cell density in LPS-exposed preterm pigs

The pro-inflammatory cytokines IL-1β and IL-8 and the anti-inflammatory cytokine IL-10 were evaluated in the distal small intestinal tissues. IL-1β levels tended to be lower in COL vs. FOR pigs ($P = 0.06$, Fig. 5A), and IL-8 levels in COL and CGMP were lower than in FOR pigs ($P < 0.001$ and $P = 0.09$, respectively, Fig.5B). No differences in IL-10 levels were observed (Fig. 5C). I-FABP, a gut maturation marker, showed a tendency to be elevated in the distal small intestine of COL pigs (but not OPN nor CGMP pigs), relative to FOR pigs ($P = 0.06$, Fig. 5D). Both colon and distal small intestinal mucin-containing goblet cell densities were significantly higher in COL pigs than FOR pigs ($P < 0.05$, Fig. 5E-F) with intermediate values in OPN and CGMP pigs.

Blood biochemistry and systemic immunity in LPS-exposed preterm pigs

Comparing serum biochemical parameters at euthanasia on day 5, COL pigs had higher levels of cholesterol, phosphate, urea nitrogen, Mg and K than FOR pigs (all $P < 0.05$, Table 2). CGMP-enriched formula-fed pigs had 2.4-fold higher levels of urea nitrogen ($P < 0.001$), and tended to have increased serum levels of P ($P = 0.07$) and Na ($P = 0.08$), relative to FOR pigs (Table 2). Systemic parameters on d 5 modulated by LPS including albumin, ALT (Table 2), glucose and IL-1β (Fig. 6A-B) were not different among the feeding groups while serum Fe decreased in COL pigs (relative to FOR pigs) to the basal levels found in pigs without LPS exposure ($P < 0.05$, Fig. 6C). Total blood leukocyte, neutrophil, lymphocyte and monocyte counts on d 5 were not different among the groups (Table 2).

On day 3, COL pigs had higher frequencies of blood helper T cells ($CD3^+CD4^+CD8^-$ lymphocytes) than FOR pigs ($P < 0.05$), whereas expression levels of CD4 on blood helper T cells tended to be lower in COL and

OPN pigs than in FOR pigs (P = 0.07 and 0.06, respectively, Fig. 6D-E). *In vitro* blood neutrophil phagocytic capacity against *E.coli* on day 5 showed tendency to be lower in COL vs. FOR pigs (P = 0.07) while OPN and CGMP pigs showed similar levels to that in FOR pigs (Fig. 6F).

Colon microbiota composition in LPS-exposed preterm pigs

High quality reads were merged and aligned to 1098 zOTUs, then classified into 55 bacterial groups at genus level (28 identified genera and 27 unambiguously identified at upper levels). The numbers of observed OTU (alpha diversity) were similar among the groups (Fig. 7A). *Enterococcus*, *Streptococcus*, *Klebsiella*, *Clostridium* and spp. and members of Enterobacteriaceae, Clostridiaceae were dominant groups in the colon content without significant differences in abundance of any bacterial groups among the four treatments (Fig. 7B). Using PCoA analysis for both unweighted and weighted Unifract distance matrix, no difference was detected among the four groups (Fig. 7C-D). No specific bacterial groups were significantly affected by COL, OPN or CGMP intervention, relative to FOR.

***In vitro* cell studies**

We used porcine IPEC-J2 cell line to test the *in vitro* effects of CGMP and OPN on IEC proliferation and related cytokine secretion. Among tested protein/peptide concentrations (0, 0.01, 0.1 and 1 g/L), CGMP did not stimulate IEC proliferation. In contrast, OPN increased cell proliferation in a dose-dependent manner with the highest proliferation of approximately 150% at 1 g/L (Fig. 8A).

For the cytokine secretion assay, cell stimulation with CGMP or OPN alone slightly down-regulated the levels of IL-8, relative to control cells cultured in the same serum-free medium (P = 0.06 and P < 0.05, respectively; Fig. 8B). LPS stimulation led to a 1.7-fold increase in IL-8 levels (P < 0.001; Fig. 8B). Co-stimulation with LPS and CGMP, but not LPS and OPN, decreased IL-8 secretion, relative to LPS stimulation alone (P < 0.01; Fig. 8B).

Discussion

Clinical studies indicate that prenatal inflammation may have a variety of effects on the preterm neonate and its organs, depending on the nature, timing and length of fetal inflammatory exposure (52). Studies in fetal lambs also support this conclusion (26, 34, 57), but these studies did not allow rearing of preterm lambs exposed to prenatal inflammation, to investigate clinical effects after birth and postnatal feeding, such as NEC. In our previous preterm pig study (33), we induced prenatal inflammation by an intra-amniotic LPS injection three days before preterm birth and found that IA LPS led to strong fetal gut immune responses and increased postnatal diarrhea and symptoms of systemic inflammation when preterm pigs were fed infant formula. However, the postnatal NEC outcomes were not affected by IA LPS. In this study, we first confirmed the most important effects of IA LPS in formula-fed pigs on postnatal day 5, including increased systemic inflammation and decreased gut enzyme activities in LPS vs. CON pigs, with no impact on NEC incidence. Then we aimed to dampen these detrimental effects of IA LPS by feeding bioactive milk diets. We found that colostrum feeding reduced diarrhea and NEC severity, lowered levels of gut inflammatory cytokines, and increased gut enzyme activities and goblet cell density. Enrichment of formula with CGMP and OPN exerted some beneficial effects (reduced diarrhea and gut cytokines, increased gut lactase activity) and the results were supported by proliferative and anti-inflammatory properties of CGMP and OPN *in vitro*. However, we conclude that in the presence of prenatal inflammation, the *in vivo* effects of these interventions were modest, relative to our previous studies on preterm pigs not subjected to prenatal inflammation (31, 48). Possibly, exposure to inflammation before birth makes the preterm newborn neonate less sensitive to the immune-modulatory effects of bioactive milk diets after birth.

For those preterm infants with insufficient or delayed intake of mother's own milk, intact bovine colostrum may be a better alternative than processed infant formulas as it contains high levels of multiple bioactive components, including IgG, lactoferrin and OPN (10). In recent years, multiple preterm pig studies have showed positive effects of bovine colostrum to improve gut function and maturation, as well as to

304 decrease gut inflammatory responses (20, 48). In addition, bovine colostrum also reduced the time to full
305 enteral feeding and increased protein intake in preliminary studies in preterm infants, relative to infant
306 formula (22). This preliminary infant study did not allow us to evaluate if the postnatal effects of colostrum
307 feeding may depend on the inflammatory status at birth and previous animal studies with colostrum have
308 not addressed this research question. Now we demonstrate that in preterm pigs exposed to IA
309 inflammation, bovine colostrum feeding reduces or has tendency to reduce diarrhea, NEC severity and gut
310 inflammatory cytokine levels, reflecting an anti-inflammatory activity. For some of the parameters such as
311 NEC severity and gut IL1-β, the tendency may have become significant if the study sample size was greater.
312 Nevertheless, the effects of colostrum in this current study were much less pronounced than a previous
313 study using similar products and feeding regimes but without prenatal inflammation (e.g. clear colostrum
314 protective effects on NEC lesions in all regions, gut nutrient absorption, permeability, mucosal morphology
315 and tissue cytokines) (48). This suggests that adverse inflammatory conditions before birth may lead to a
316 less responsive gut status to immune-modulatory diets after birth. An immune-modulatory diet, like
317 colostrum, may be most effective in a state where the gut has not already been challenged with pro-
318 inflammatory stimuli before birth.

319 In addition to gut effects, some systemic immune parameters were also modulated by colostrum
320 feeding, including the increased percentage of blood T-helper cells, as well as decreased blood neutrophil
321 phagocytosis function, relative to formula feeding. Increased percentage of blood T-helper cells were
322 associated with the immune maturation over the first two weeks of life in preterm pigs (40), suggesting
323 systemic immune maturational effects of bovine colostrum in the current study. In contrast, decreased
324 neutrophil phagocytic capacity may relate to the numerical increase in blood neutrophil counts in COL vs.
325 FOR pigs, as newly recruited neutrophils from the bone marrow to the circulation may have a relatively less
326 mature function. It remains to be elucidated however, if COL pigs have an improved systemic immune
327 competence and infection resistance, relative to FOR pigs. Further, colostrum feeding in LPS-exposed pigs
328 reversed levels of several parameters including serum iron and gut lactase activity to levels found in

329 formula-fed pigs without prenatal LPS challenge. These effects may be derived from both anti-inflammatory
330 and nutritional properties of colostrum (relative to formula). The different nutritional composition between
331 colostrum and formula may also explain differences in serum levels of BUN, P, Mg and K.

332 CGMP and OPN have been reported to exert immune-modulatory functions both *in vivo* and *in vitro*
333 (18, 29, 41, 55). In the current study, we showed proliferative effects of OPN and anti-inflammatory
334 functions of CGMP in IECs *in vitro*, coupled with some moderate improvements of gut functions in preterm
335 pigs (e.g. less diarrhea and inflammatory cytokines and higher lactase activity). For CGMP, a previous *in*
336 *vitro* study using an IEC cell line demonstrated that CGMP decreased inflammatory gene expressions
337 including *IL1B*, *IL8* and *TNFA* (18). On the other hand, the *in vivo* beneficial trends of OPN in this study was
338 very modest (only weak tendency of reduced diarrhea), in contrast with a previous study showing
339 significant effects of OPN to reduce NEC severity and gut inflammation in preterm pigs without prenatal
340 inflammation (31). Strong innate immune modulations *in vitro* and modest *in vivo* effects support our
341 speculation that effects of bioactive milk proteins after birth is less pronounced if the newborn has already
342 been exposed to prenatal inflammation. Similar to colostrum, CGMP- and OPN- supplemented formula
343 diets may be more effective to prevent feeding-induced inflammatory reactions than to act as a therapy
344 when gut inflammation is already present due to prenatal inflammatory stimuli.

345 Interestingly, none of the diet interventions led to changes in the gut microbiota composition or
346 diversity, relative to infant formula. Both CGMP and OPN have previously been reported to modulate
347 microbiota composition and play a role in maintaining gut bacterial homeostasis (19, 35). In preterm pigs
348 without prenatal inflammation, colostrum feeding also reduced the abundance of *Enterococcus* spp. (56), a
349 bacterial genus that is often associated with increased gut inflammation (4). As indicated in a previous pig
350 study (33), fetal LPS-induced gut inflammation may alter bacterial gut colonization during early life, thereby
351 affecting the host-microbiota interactions and responses to nutrition. Similar abundance of *Enterococcus*

spp. in all four groups in this study also supports that the effects of colostrum, CGMP and OPN supplementation were relatively mild.

In conclusion, milk diets containing bioactive components such as bovine colostrum, OPN- or CGMP-enriched formulas exerted modest protective effects on the immature gut in preterm pigs exposed to prenatal inflammation. Possibly, prenatal inflammation inhibits the protective effects of milk bioactive diets. Potentially, the postnatal effects of prenatal inflammation are highly dependent on the nature, timing and length of the inflammatory insult. Thus, preterm neonates exposed to prenatal inflammation may require careful evaluation and highly individualized clinical care and diets. Further investigations are required to define the optimal nutritional regimen for preterm infants subjected to prenatal inflammation, especially when mother´s own milk is lacking or absent.

Acknowledgement

The authors would like to thank Elin Skytte, Jane Polvsen and Kristina Møller for experimental and laboratory assistance. The study was supported by STIMMUNE project funded by the center of Arla Food for Health, NEOCOL project funded by Innovation Foundation Denmark, and Biofiber Damino, Denmark. Biofiber Damino and the center of Arla Food for Health had no impacts on the study design, experiment, data analysis and manuscript writing. University of Copenhagen holds a patent on bovine colostrum use for pediatric patients. PTS is listed as the sole inventor but has declined any share of potential revenue arising from commercial exploitation of such a patent. The remaining authors have no potential conflicts of interest.

References

1. **Andrews WW, Goldenberg RL, Faye-Petersen O, Cliver S, Goepfert AR, and Hauth JC**. The Alabama Preterm Birth study: polymorphonuclear and mononuclear cell placental infiltrations, other markers of inflammation, and outcomes in 23- to 32-week preterm newborn infants. *Am J Obstet Gynecol* 195: 803-808, 2006.

2. **Arayici S, Kadioglu Simsek G, Oncel MY, Eras Z, Canpolat FE, Oguz SS, Uras N, Zergeroglu S, and Dilmen U**. The effect of histological chorioamnionitis on the short-term outcome of preterm infants </=32 weeks: a single-center study. *The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstet* 27: 1129-1133, 2014.

3. **Been JV, Lievense S, Zimmermann LJ, Kramer BW, and Wolfs TG**. Chorioamnionitis as a risk factor for necrotizing enterocolitis: a systematic review and meta-analysis. *The Journal of pediatrics* 162: 236-242.e232, 2013.

4. **Birck MM, Nguyen DN, Cilieborg MS, Kamal SS, Nielsen DS, Damborg P, Olsen JE, Lauridsen C, Sangild PT, and Thymann T**. Enteral but not parenteral antibiotics enhance gut function and prevent necrotizing enterocolitis in formula-fed newborn preterm pigs. *Am J Physiol Gastrointest Liver Physiol* 310: G323-333, 2016.

5. **Blencowe H, Cousens S, Chou D, Oestergaard M, Say L, Moller AB, Kinney M, and Lawn J**. Born too soon: the global epidemiology of 15 million preterm births. *Reproductive health* 10 Suppl 1: S2, 2013.

6. **Blencowe H, Cousens S, Oestergaard MZ, Chou D, Moller A-B, Narwal R, Adler A, Vera Garcia C, Rohde S, Say L, and Lawn JE**. National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. *The Lancet* 379: 2162-2172, 2012.

7. **Bouhallab S, Favrot C, and Maubois J, L.** Growth-promoting activity of tryptic digest of caseinomacropeptide for Lactococcus lactis subsp lactis. *Lait* 73: 73-77, 1993.

8. **Brody EP**. Biological activities of bovine glycomacropeptide. *Br J Nutr* 84 Suppl 1: S39-46, 2000.

9. **Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, and Knight R**. QIIME allows analysis of high-throughput community sequencing data. *Nature methods* 7: 335-336, 2010.

10. **Chatterton DE, Nguyen DN, Bering SB, and Sangild PT**. Anti-inflammatory mechanisms of bioactive milk proteins in the intestine of newborns. *The international journal of biochemistry & cell biology* 45: 1730-1747, 2013.

11. **Cristofalo EA, Schanler RJ, Blanco CL, Sullivan S, Trawoeger R, Kiechl-Kohlendorfer U, Dudell G, Rechtman DJ, Lee ML, Lucas A, and Abrams S**. Randomized trial of exclusive human milk versus preterm formula diets in extremely premature infants. *The Journal of pediatrics* 163: 1592-1595 e1591, 2013.

12. **Edgar R**. UCHIME2: improved chimera prediction for amplicon sequencing. *bioRxiv* 2016.

13. **Edgar RC**. UNOISE2: improved error-correction for Illumina 16S and ITS amplicon sequencing. *bioRxiv* 2016.

14. **Erdemir G, Kultursay N, Calkavur S, Zekioglu O, Koroglu OA, Cakmak B, Yalaz M, Akisu M, and Sagol S**. Histological chorioamnionitis: effects on premature delivery and neonatal prognosis. *Pediatrics and neonatology* 54: 267-274, 2013.

15. **Ericson JE, and Laughon MM**. Chorioamnionitis: implications for the neonate. *Clin Perinatol* 42: 155-165, ix, 2015.

16. **Gravett MG, Rubens CE, and Nunes TM**. Global report on preterm birth and stillbirth (2 of 7): discovery science. *BMC pregnancy and childbirth* 10 Suppl 1: S2, 2010.

17. **Gustavo Hermes R, Molist F, Francisco Perez J, Gomez de Segura A, Ywazaki M, Davin R, Nofrarias M, Korhonen TK, Virkola R, and Martin-Orue SM**. Casein glycomacropeptide in the diet may reduce Escherichia coli attachment to the intestinal mucosa and increase the intestinal lactobacilli of early weaned piglets after an enterotoxigenic E. coli K88 challenge. *Br J Nutr* 109: 1001-1012, 2013.

18. **Hermes RG, Manzanilla EG, Martin-Orue SM, Perez JF, and Klasing KC**. Influence of dietary ingredients on in vitro inflammatory response of intestinal porcine epithelial cells challenged by an enterotoxigenic Escherichia coli (K88). *Comparative immunology, microbiology and infectious diseases* 34: 479-488, 2011.

19. **Ito K, Nakajima A, Fukushima Y, Suzuki K, Sakamoto K, Hamazaki Y, Ogasawara K, Minato N, and Hattori M**. The potential role of Osteopontin in the maintenance of commensal bacteria homeostasis in the intestine. *PloS one* 12: e0173629, 2017.

20. **Jensen ML, Sangild PT, Lykke M, Schmidt M, Boye M, Jensen BB, and Thymann T**. Similar efficacy of human banked milk and bovine colostrum to decrease incidence of necrotizing enterocolitis in preterm piglets. *Am J Physiol Regul Integr Comp Physiol* 305: R4-R12, 2013.

21. **Jensen ML, Thymann T, Cilieborg MS, Lykke M, Mølbak L, Jensen BB, Schmidt M, Kelly D, Mulder I, Burrin DG, and Sangild PT**. Antibiotics modulate intestinal immunity and prevent necrotizing enterocolitis in preterm neonatal piglets. *Am J Physiol Gastrointest Liver Physiol* 306: G59-71, 2014.

22. **Juhl SM, Ye X, Zhou P, Li Y, Iyore EO, Zhang L, Jiang P, van Goudoever JB, Greisen G, and Sangild PT**. Bovine Colostrum for Preterm Infants in the First Days of Life: A Randomized Controlled Pilot Trial. *J Pediatr Gastroenterol Nutr* 66: 471-478, 2018.

23. **Koh A, da Silva AP, Bansal AK, Bansal M, Sun C, Lee H, Glogauer M, Sodek J, and Zohar R**. Role of osteopontin in neutrophil function. *Immunology* 122: 466-475, 2007.

24. **Korbage de Araujo MC, Schultz R, do Rosario Dias de Oliveira L, Ramos JL, and Vaz FA**. A risk factor for early-onset infection in premature newborns: invasion of chorioamniotic tissues by leukocytes. *Early human development* 56: 1-15, 1999.

25. **Kramer BW**. Chorioamnionitis - new ideas from experimental models. *Neonatology* 99: 320-325, 2011.

26. **Kramer BW, Ikegami M, Moss TJM, Nitsos I, Newnham JP, and Jobe AH**. Endotoxin-induced Chorioamnionitis Modulates Innate Immunity of Monocytes in Preterm Sheep. *American Journal of Respiratory and Critical Care Medicine* 171: 73-77, 2005.

27. **Krych L, Kot W, Bendtsen KMB, Hansen AK, Vogensen FK, and Nielsen DS**. Have you tried spermine? A rapid and cost-effective method to eliminate dextran sodium sulfate inhibition of PCR and RT-PCR. *Journal of microbiological methods* 144: 1-7, 2018.

28. **Li Y, Nguyen DN, Obelitz-Ryom K, Andersen AD, Thymann T, Chatterton DEW, Purup S, Heckmann AB, Bering SB, and Sangild PT**. Bioactive Whey Protein Concentrate and Lactose Stimulate Gut Function in Formula-fed Preterm Pigs. *J Pediatr Gastroenterol Nutr* 66: 128-134, 2018.

29. **Lonnerdal B**. Human Milk: Bioactive Proteins/Peptides and Functional Properties. *Nestle Nutr Inst Workshop Ser* 86: 97-107, 2016.

30. **Mandal S, Van Treuren W, White RA, Eggesbo M, Knight R, and Peddada SD**. Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microbial ecology in health and disease* 26: 27663, 2015.

31. **Moller HK, Thymann T, Fink LN, Frokiaer H, Kvistgaard AS, and Sangild PT**. Bovine colostrum is superior to enriched formulas in stimulating intestinal function and necrotising enterocolitis resistance in preterm pigs. *Br J Nutr* 105: 44-53, 2011.

32. **Nguyen DN, Jiang P, Frokiaer H, Heegaard PM, Thymann T, and Sangild PT**. Delayed development of systemic immunity in preterm pigs as a model for preterm infants. *Sci Rep* 6: 36816, 2016.

33. **Nguyen DN, Thymann T, Goericke-Pesch SK, Ren S, Wei W, Skovgaard K, Damborg P, Brunse A, van Gorp C, Kramer BW, Wolfs TG, and Sangild PT**. Prenatal Intra-Amniotic Endotoxin Induces Fetal Gut and Lung Immune Responses and Postnatal Systemic Inflammation in Preterm Pigs. *The American journal of pathology* 188: 2629-2643, 2018.

34. **Nikiforou M, Jacobs EM, Kemp MW, Hornef MW, Payne MS, Saito M, Newnham JP, Janssen LE, Jobe AH, Kallapur SG, Kramer BW, and Wolfs TG**. Intra-amniotic Candida albicans infection induces mucosal injury and inflammation in the ovine fetal intestine. *Sci Rep* 6: 29806, 2016.

35. **Ntemiri A, Chonchuir FN, O'Callaghan TF, Stanton C, Ross RP, and O'Toole PW**. Glycomacropeptide Sustains Microbiota Diversity and Promotes Specific Taxa in an Artificial Colon Model of Elderly Gut Microbiota. *Journal of agricultural and food chemistry* 65: 1836-1846, 2017.

36. **Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'hara R, Simpson GL, Solymos P, Stevens MHH, and Wagner H**. Package 'vegan'. *Community ecology package, version* 2: 2013.

37. **Ostergaard MV, Cilieborg MS, Skovgaard K, Schmidt M, Sangild PT, and Bering SB**. Preterm Birth Reduces Nutrient Absorption With Limited Effect on Immune Gene Expression and Gut Colonization in Pigs. *J Pediatr Gastroenterol Nutr* 61: 481-490, 2015.

38. **Patarca R, Saavedra RA, and Cantor H**. Molecular and cellular basis of genetic resistance to bacterial infection: the role of the early T-lymphocyte activation-1/osteopontin gene. *Critical reviews in immunology* 13: 225-246, 1993.

39. **Paulson JN, Stine OC, Bravo HC, and Pop M**. Differential abundance analysis for microbial marker-gene surveys. *Nature methods* 10: 1200-1202, 2013.

40. **Ren S, Hui Y, Obelitz-Ryom K, Brandt AB, Kot W, Nielsen DS, Thymann T, Sangild PT, and Nguyen DN**. Neonatal gut and immune maturation is determined more by postnatal age than by post-conceptional age in moderately preterm pigs. *Am J Physiol Gastrointest Liver Physiol* 2018.

41. **Requena P, Gonzalez R, Lopez-Posadas R, Abadia-Molina A, Suarez MD, Zarzuelo A, de Medina FS, and Martinez-Augustin O**. The intestinal antiinflammatory agent glycomacropeptide has immunomodulatory actions on rat splenocytes. *Biochemical pharmacology* 79: 1797-1804, 2010.

42. **Robitaille G, and Champagne CP**. Growth-promoting effects of pepsin- and trypsin-treated caseinomacropeptide from bovine milk on probiotics. *The Journal of dairy research* 81: 319-324, 2014.

43. **Rognes T, Flouri T, Nichols B, Quince C, and Mahe F**. VSEARCH: a versatile open source tool for metagenomics. *PeerJ* 4: e2584, 2016.

44. **Sangild PT, Petersen YM, Schmidt M, Elnif J, Petersen TK, Buddington RK, Greisen G, Michaelsen KF, and Burrin DG**. Preterm birth affects the intestinal response to parenteral and enteral nutrition in newborn pigs. *The Journal of nutrition* 132: 3786-3794, 2002.

45. **Sangild PT, Siggers RH, Schmidt M, Elnif J, Bjornvad CR, Thymann T, Grondahl ML, Hansen AK, Jensen SK, Boye M, Moelbak L, Buddington RK, Westrom BR, Holst JJ, and Burrin DG**. Diet- and colonization-dependent intestinal dysfunction predisposes to necrotizing enterocolitis in preterm pigs. *Gastroenterology* 130: 1776-1792, 2006.

46. **Sangild PT, Sjostrom H, Noren O, Fowden AL, and Silver M**. The prenatal development and glucocorticoid control of brush-border hydrolases in the pig small intestine. *Pediatric research* 37: 207-212, 1995.

47. **Shen RL, Pontoppidan PE, Rathe M, Jiang P, Hansen CF, Buddington RK, Heegaard PM, Muller K, and Sangild PT**. Milk diets influence doxorubicin-induced intestinal toxicity in piglets. *Am J Physiol Gastrointest Liver Physiol* 311: G324-333, 2016.

48. **Shen RL, Thymann T, Ostergaard MV, Stoy AC, Krych L, Nielsen DS, Lauridsen C, Hartmann B, Holst JJ, Burrin DG, and Sangild PT**. Early gradual feeding with bovine colostrum improves gut function and NEC resistance relative to infant formula in preterm pigs. *Am J Physiol Gastrointest Liver Physiol* 309: G310-323, 2015.

514 49. **Siggers RH, Siggers J, Thymann T, Boye M, and Sangild PT**. Nutritional modulation of the gut
515 microbiota and immune system in preterm neonates susceptible to necrotizing enterocolitis. *The Journal of*
516 *nutritional biochemistry* 22: 511-521, 2011.
517 50. **Snyder CC, Wolfe KB, Gisslen T, Knox CL, Kemp MW, Kramer BW, Newnham JP, Jobe AH,**
518 **and Kallapur SG**. Modulation of lipopolysaccharide-induced chorioamnionitis by Ureaplasma parvum in
519 sheep. *American Journal of Obstetrics and Gynecology* 208: 399.e391-399.e398, 2013.
520 51. **Solomons NW**. Modulation of the immune system and the response against pathogens with
521 bovine colostrum concentrates. *European journal of clinical nutrition* 56 Suppl 3: S24-28, 2002.
522 52. **Sweeney EL, Kallapur SG, Meawad S, Gisslen T, Stephenson SA, Jobe AH, and Knox CL**.
523 Ureaplasma Species Multiple Banded Antigen (MBA) Variation Is Associated with the Severity of
524 Inflammation In vivo and In vitro in Human Placentae. *Frontiers in cellular and infection microbiology* 7: 123,
525 2017.
526 53. **Underwood MA**. Human milk for the premature infant. *Pediatric clinics of North America* 60:
527 189-207, 2013.
528 54. **Wang KX, and Denhardt DT**. Osteopontin: role in immune regulation and stress responses.
529 *Cytokine & growth factor reviews* 19: 333-345, 2008.
530 55. **West CE, Kvistgaard AS, Peerson JM, Donovan SM, Peng YM, and Lonnerdal B**. Effects of
531 osteopontin-enriched formula on lymphocyte subsets in the first 6 months of life: a randomized controlled
532 trial. *Pediatric research* 82: 63-71, 2017.
533 56. **Willems R, Krych L, Rybicki V, Jiang P, Sangild PT, Shen RL, Hensel KO, Wirth S, Postberg J,**
534 **and Jenke AC**. Introducing enteral feeding induces intestinal subclinical inflammation and respective
535 chromatin changes in preterm pigs. *Epigenomics* 7: 553-565, 2015.
536 57. **Wolfs TG, Kallapur SG, Knox CL, Thuijls G, Nitsos I, Polglase GR, Collins JJ, Kroon E, Spierings**
537 **J, Shroyer NF, Newnham JP, Jobe AH, and Kramer BW**. Antenatal ureaplasma infection impairs
538 development of the fetal ovine gut in an IL-1-dependent manner. *Mucosal immunology* 6: 547-556, 2013.

58. **Ylijoki M, Ekholm E, Haataja L, Lehtonen L, and group Ps**. Is chorioamnionitis harmful for the brain of preterm infants? A clinical overview. *Acta Obstet Gynecol Scand* 91: 403-419, 2012.

59. **Yoon BH, Romero R, Kim M, Kim EC, Kim T, Park JS, and Jun JK**. Clinical implications of detection of Ureaplasma urealyticum in the amniotic cavity with the polymerase chain reaction. *Am J Obstet Gynecol* 183: 1130-1137, 2000.

60. **Zhu B, Suzuki K, Goldberg HA, Rittling SR, Denhardt DT, McCulloch CA, and Sodek J**. Osteopontin modulates CD44-dependent chemotaxis of peritoneal macrophages through G-protein-coupled receptors: evidence of a role for an intracellular form of osteopontin. *Journal of cellular physiology* 198: 155-167, 2004.

549 **Table 1. Nutritional compositions in the four diets used in preterm pigs**

	Formula	CGMP-Formula	OPN-Formula	Colostrum
Protein (g/L)	40.6	70.6	42.8	67.6
Carbohydrate (g/L)	45.0	34.9	45	26.8
Lipid (g/L)	41.3	31.5	41.3	36.8
Energy (kJ/L)	3017.0	3016.9	3054.7	3004.7

550

551 **Table 2. Blood biochemistry and hematology on d 5 in preterm pigs born with prenatal inflammation**

	FOR (n=11)	COL (n=10)	CGMP (n=11)	OPN (n=12)
Total protein (g/L)	30.0±0.8	31.8±1.2	31.4±1.6	29.9±0.9
Albumin (g/L)	11.1±0.4	11.9±0.6	12.2±0.7	11.1±0.4
ALT (U/L)	18.6±0.6	20.4±0.7	23.7±3.6	18.3±0.9
Cholesterol (mmol/L)	2.4±0.1	3.0±0.2*	2.4±0.2	2.6±0.1
Iron (µmol/L)	6.2±0.8	4.5±1.4*	3.6±0.4	4.0±0.7
Phosphate (mmol/L)	1.5±0.1	1.9±0.1**	1.7±0.1$^{\#}$	1.6±0.1
Urea nitrogen (mmol/L)	3.5±0.6	11.4±0.7***	8.4±0.9***	4.2±0.8
Mg++ (mmol/L)	0.80±0.03	1.05±0.05***	0.84±0.04	0.84±0.03
Na+ (mmol/L)	148.0±0.7	147.1±1.1	152.2±2.8$^{\#}$	149.3±1.9
K+ (mmol/L)	3.4±0.1	4.2±0.1***	3.6±0.1	3.6±0.2
Total leukocytes	4.3±0.5	6.8±1.3	4.2±0.6	3.5±0.5
Neutrophils	2.7±0.5	4.2±1.3	2.4±0.4	2.0±0.4
Lymphocytes	1.5±0.1	2.3±0.3	1.7±0.2	1.3±0.1
Monocytes	0.10±0.02	0.10±0.02	0.07±0.01	0.10±0.02

552 *, P < 0.05;**, P < 0.01; ***, P < 0.001, relative to FOR. $^{\#}$ P = 0.07-0.08, relative to FOR. Values (mean ± SEM)
553 in three diet treatment groups were compared with values in FOR pigs (control). ALT: Alanine
554 aminotransferase.

Figure legends

Fig. 1. Schematic overview of the animal experimental design. Fetal pigs from eight litters received either LPS injection or saline/no injection (CON) at day 103 of gestation (3 days before delivery by caesarean section). In experiment 1, a proportion of LPS and CON pigs were reared until postnatal day 5 to characterize effects of IA LPS on postnatal outcomes. In experiment 2, IA LPS-exposed pigs were randomized into 4 groups fed with 4 different diets until postnatal day 5. Exp, experiment; IA, intra-amniotic; LPS, lipopolysaccharide.

Fig. 2. Effects of intra-amniotic (IA) LPS on gut and systemic parameters in formula-fed preterm pigs at postnatal day 5. (A-B) Amniotic fluid leukocyte levels and time from birth to first stand. (C-G) Circulating levels of IL-1β, glucose, iron, albumin, and alanine aminotransferase (ALT). (H-I) NEC incidence and small intestinal lactase activity. n = 32-36 in each group for (A) and 25-26 in each group for (B-I). Values are mean ± SEM. *, $P < 0.05$. CON and LPS: control and LPS-exposed pigs.

Fig. 3. Physical activities and clinical evaluations in IA LPS-exposed preterm pigs following five days of postnatal feeding with various bioactive diets. (A) Number of pigs with first stand after the first 2 days after birth, (B) diarrhea incidence during study, (C) NEC incidence, and (D) average NEC severity score across the gastrointestinal tract. All treatment groups were used to compare to FOR group (control). n = 10-12 in each treatment group. Values are shown by mean ± SEM. *, $P < 0.05$. IA: intra-amniotic.

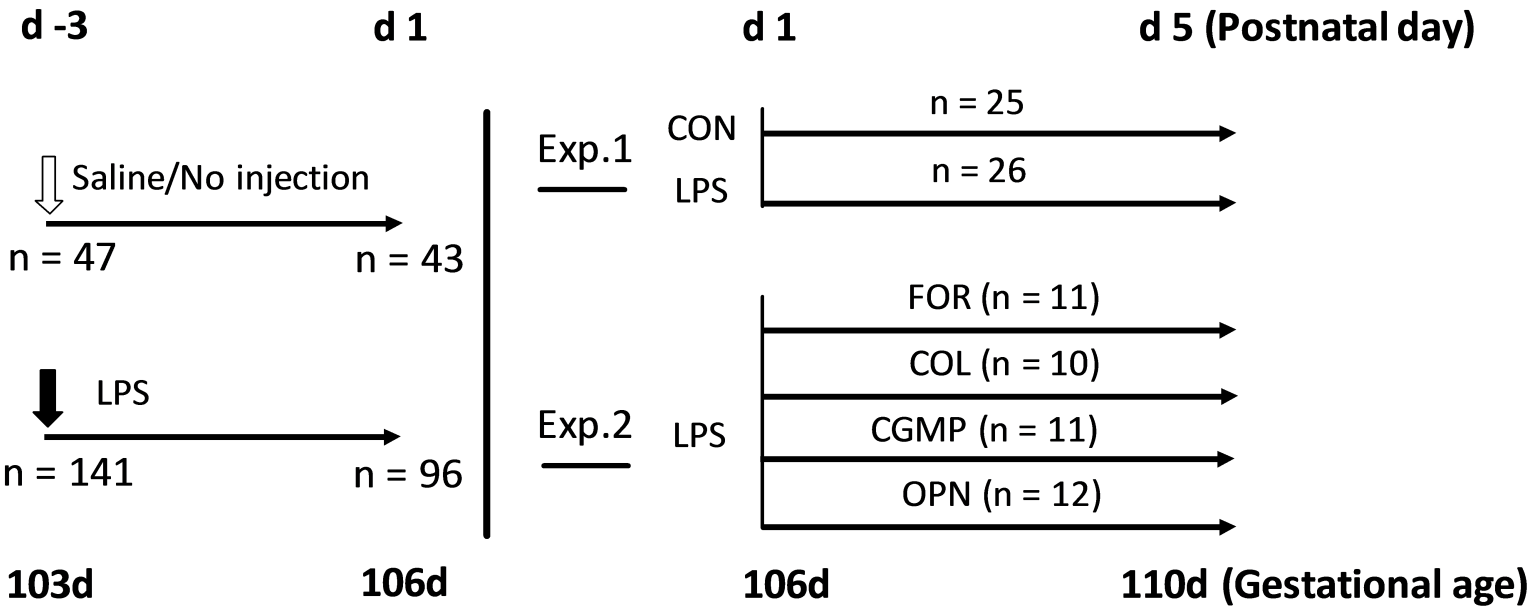
Fig. 4. Gut morphology, brush border enzymatic activities and nutrient absorption in IA LPS-exposed preterm pigs following five days of postnatal feeding with various bioactive diets. (A-C) Villus height, crypt depth and lactase activities across the three small intestinal regions. (D) Plasma galactose concentration following a galactose test. All treatment groups were compared with FOR group (control). n = 10-12 in each group for (A-C) and n = 8-10 in each group for (D). Values are mean ± SEM. *, ** and *** $P < 0.05$, 0.01 and 0.001. IA: intra-amniotic. Prox: proximal, Mid: middle, Dist: distal intestine.

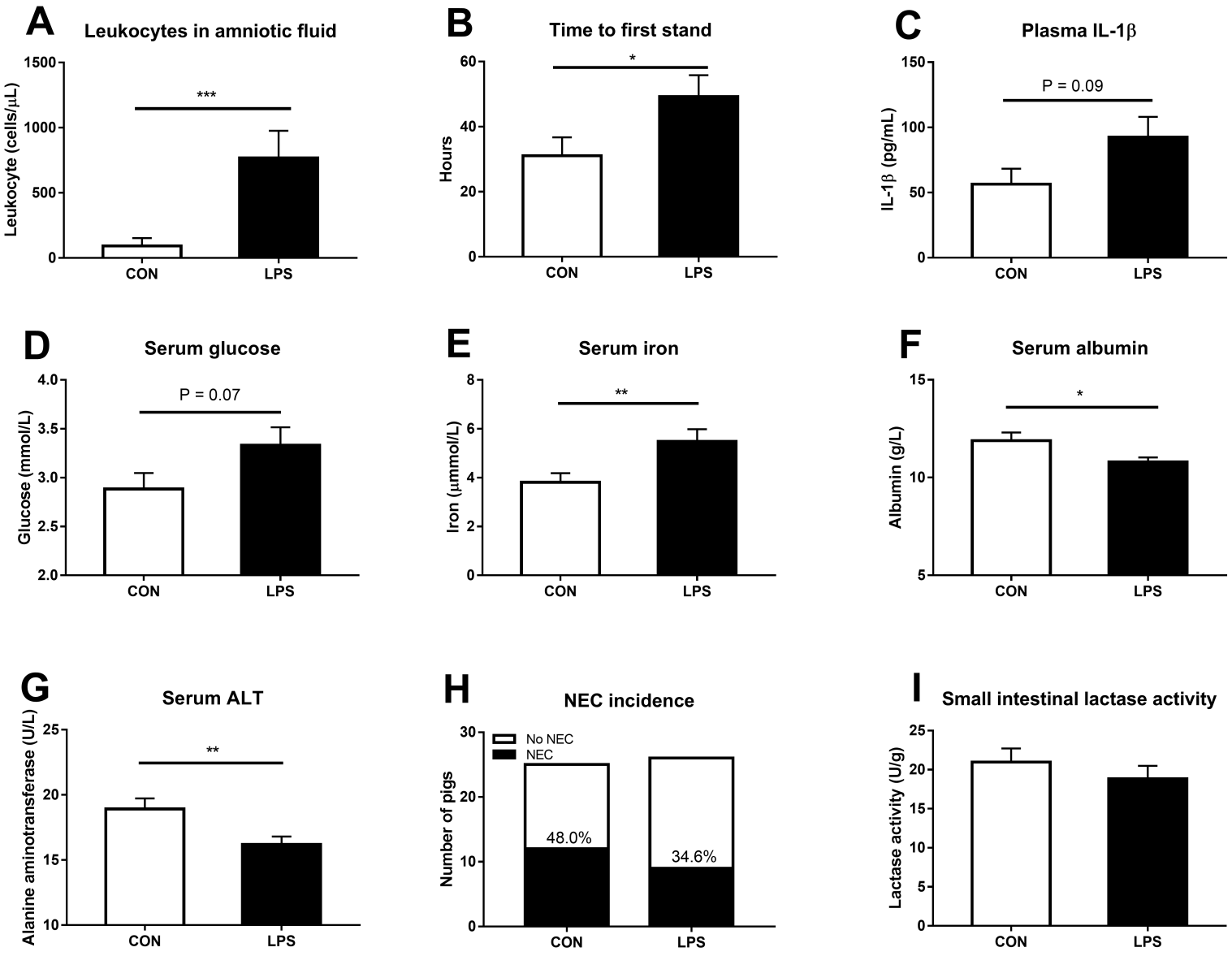
Fig. 5. Gut inflammatory cytokines and mediators and goblet cell density in IA LPS-exposed preterm pigs following five days of postnatal feeding with various bioactive diets. (A-D) Distal small intestinal IL-1β, IL-8, IL-10 and I-FABP, respectively. (E-F) Goblet cell density in distal small intestine and colon. All treatment groups compared with FOR group (control). n = 10-12 in each group. Values are mean ± SEM. * and *** P < 0.05 and 0.001. IA: intra-amniotic.

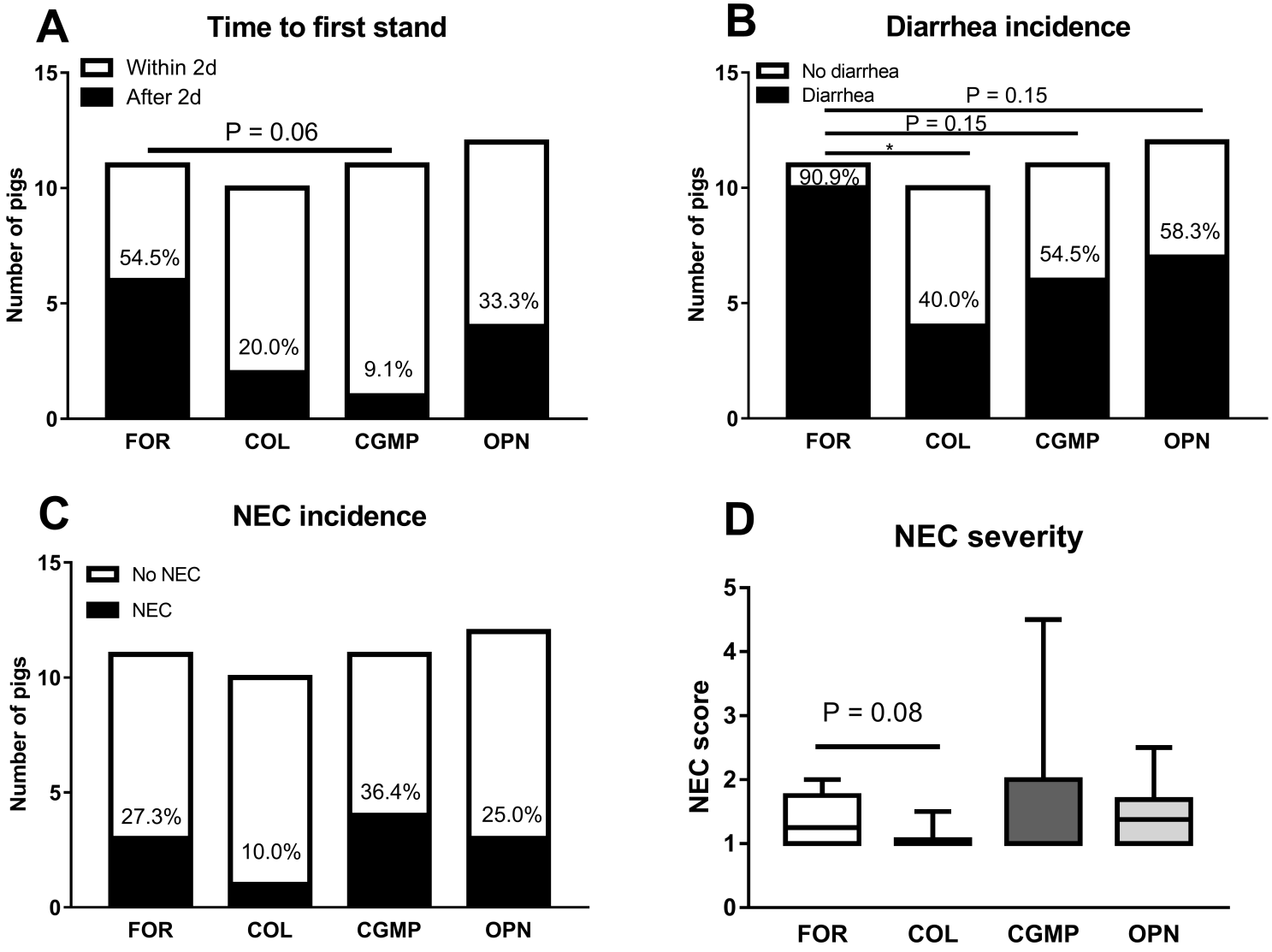
Fig. 6. Systemic immune parameters in IA LPS-exposed preterm pigs following five days of postnatal feeding with various bioactive diets. (A-C) Serum/plasma levels of glucose, IL-1β and iron at euthanasia on d 5, respectively. (D-E) Percentage of helper T cells and fluorescent intensity of CD4 on d 3. (F) Neutrophil phagocytic capacity on d 5. All treatment groups were compared with FOR group (control). n = 8-12 in each group for (A-C) and n = 5-10 in each group for (D-F). Values are mean ± SEM. *, p<0.05. IA: intra-amniotic; MFI: median fluorescent intensity

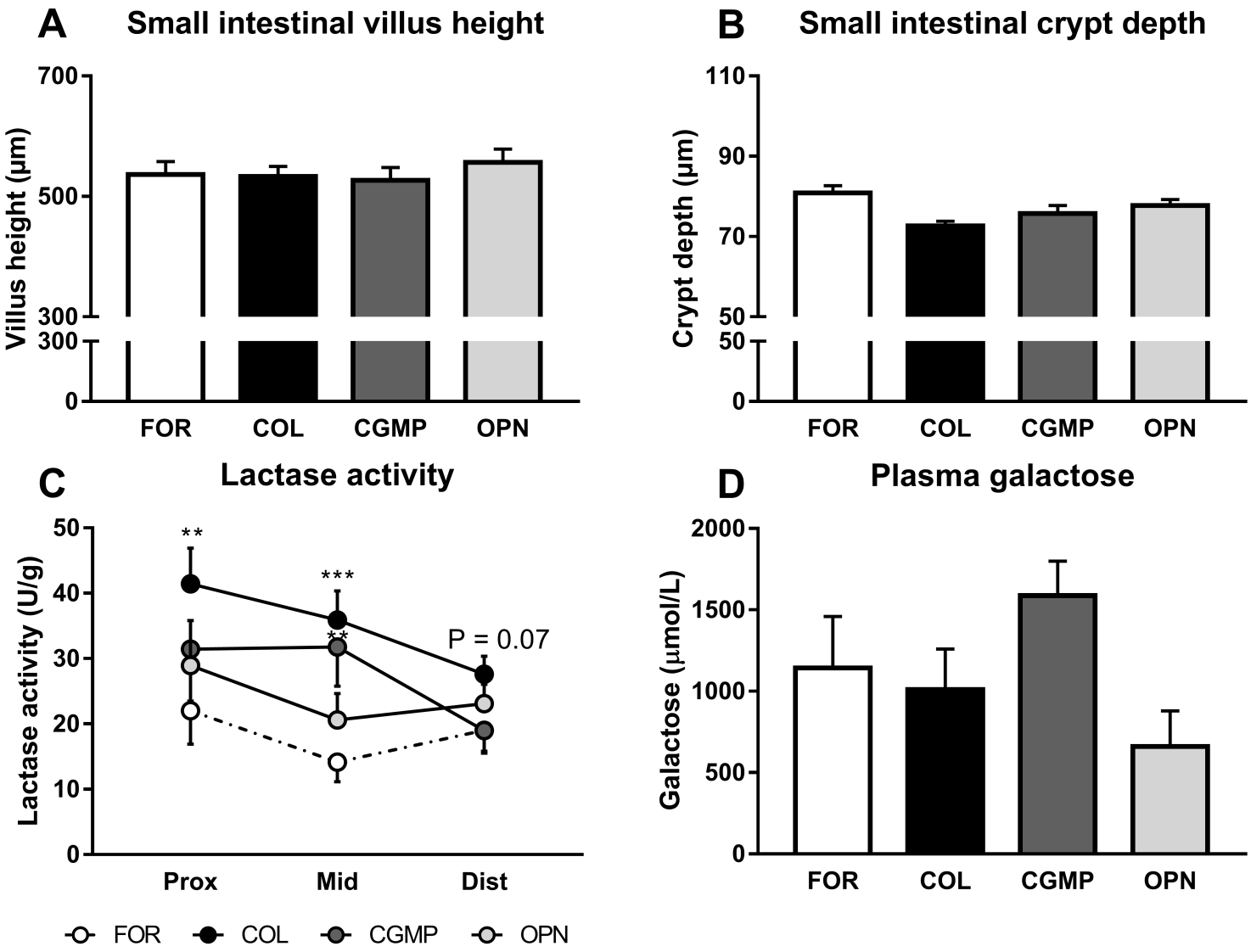
Fig. 7. Colonic microbiota composition in IA LPS-exposed preterm pigs following five days of postnatal feeding with various bioactive diets. (A) Number of operational taxonomic units (OTUs). (B) Relative abundance of specific genera, with species with an relative abundance below 1% being grouped to “others”. (C-D) Principle coordinates analysis (PCoA) plots based on unweighted and weighted Unifrac distance matrix. n = 6-10 in each group.

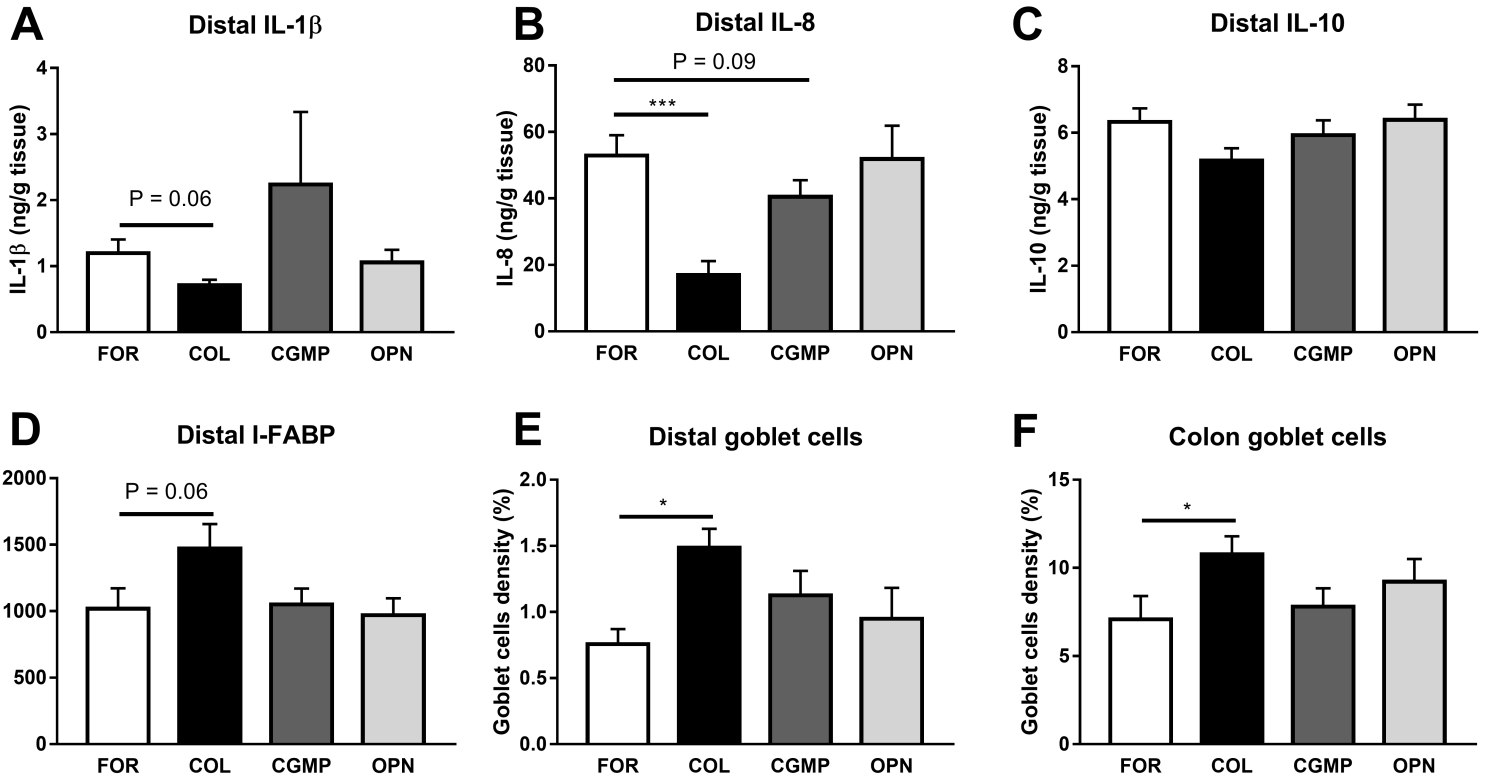
Fig. 8. *In vitro* effects of OPN and CGMP on intestinal epithelial cells (IECs). (A) *In vitro* IEC proliferation stimulated by different concentrations of CGMP and OPN (0, 0.01, 0.1 and 1 g/L) for 24 h. (B) IL-8 secretion in IECs following cell stimulation with CGMP or OPN with or without LPS presence. n = 3-4 in each treatment group. Values (mean ± SEM) not sharing the same letters are significantly different (P < 0.05). * and **, P < 0.05 and 0.01, respectively. IEC: intestinal epithelial cell.

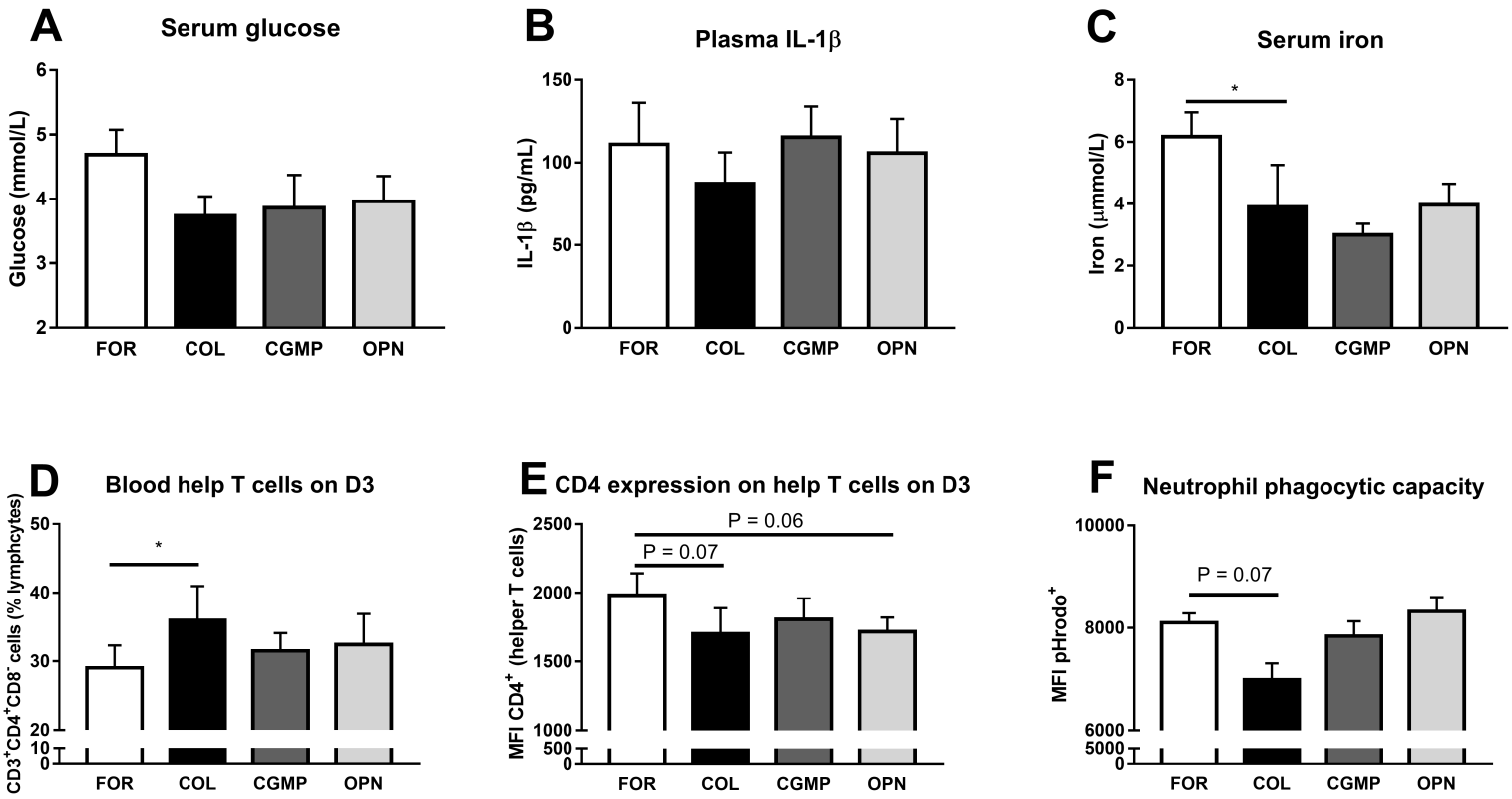

d -3
d 1
d 1
d 5 (Postnatal day)
Saline/No injection
n = 47
n = 43
LPS
n = 141
n = 96
Exp.1
CON
LPS
n = 25
n = 26
Exp.2
LPS
FOR (n = 11)
COL (n = 10)
CGMP (n = 11)
OPN (n = 12)
103d
106d
106d
110d (Gestational age)
Prenatal period
Postnatal period

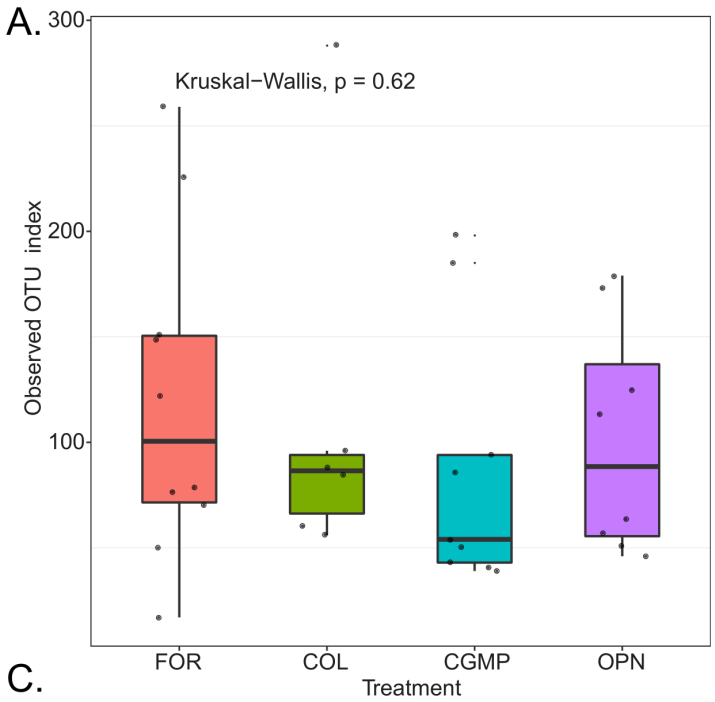

A
Leukocytes in amniotic fluid
Leukocyte (cells/μL)
1500
1000
500
0
***
CON
LPS
B
Time to first stand
Hours
60
40
20
0
*
CON
LPS
C
Plasma IL-1β
IL-1β (pg/mL)
150
100
50
0
P = 0.09
CON
LPS
D
Serum glucose
Glucose (mmol/L)
4.0
3.5
3.0
2.5
2.0
P = 0.07
CON
LPS
E
Serum iron
Iron (μmmol/L)
8
6
4
2
0
**
CON
LPS
F
Serum albumin
Albumin (g/L)
15
10
5
*
CON
LPS
G
Serum ALT
Alanine aminotransferase (U/L)
25
20
15
10
**
CON
LPS
H
NEC incidence
Number of pigs
30
20
10
0
No NEC
NEC
48.0%
34.6%
CON
LPS
I
Small intestinal lactase activity
Lactase activity (U/g)
25
20
15
10
5
0
CON
LPS

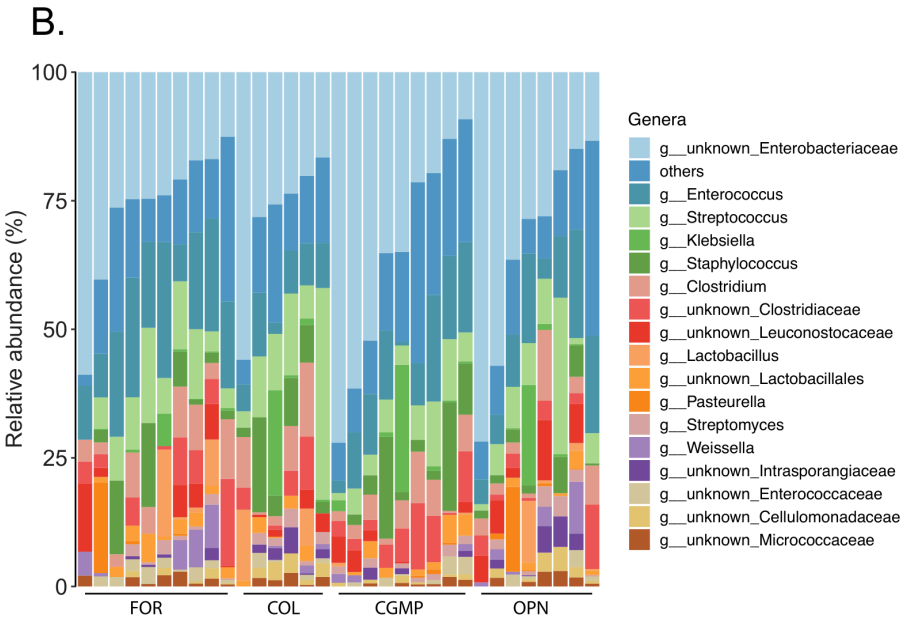

A
Time to first stand
Within 2d
After 2d
P = 0.06
Number of pigs
15
10
5
0
54.5%
20.0%
9.1%
33.3%
FOR
COL
CGMP
OPN
B
Diarrhea incidence
No diarrhea
Diarrhea
P = 0.15
P = 0.15
*
Number of pigs
90.9%
40.0%
54.5%
58.3%
FOR
COL
CGMP
OPN
C
NEC incidence
No NEC
NEC
Number of pigs
27.3%
10.0%
36.4%
25.0%
FOR
COL
CGMP
OPN
D
NEC severity
NEC score
P = 0.08
5
4
3
2
1
0
FOR
COL
CGMP
OPN

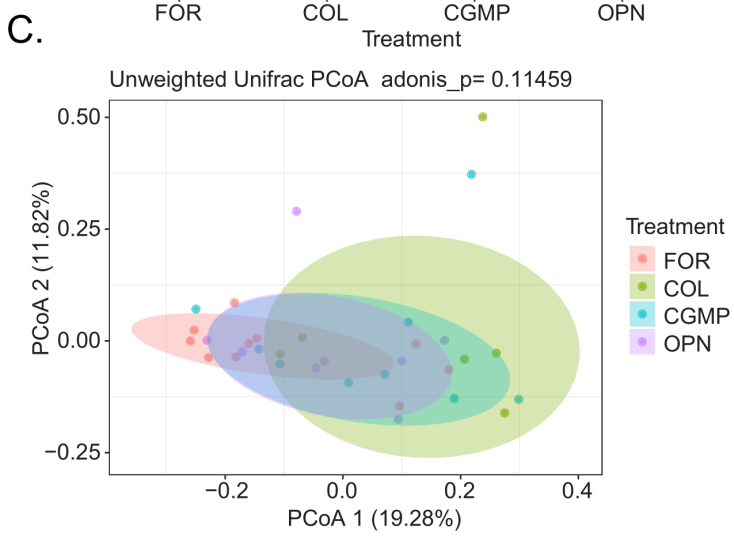

A
Small intestinal villus height
Villus height (µm)
700
500
300
300
0
FOR
COL
CGMP
OPN
B
Small intestinal crypt depth
Crypt depth (µm)
110
90
70
50
50
0
FOR
COL
CGMP
OPN
C
Lactase activity
Lactase activity (U/g)
50
40
30
20
10
0
**
***
**
P = 0.07
Prox
Mid
Dist
FOR
COL
CGMP
OPN
D
Plasma galactose
Galactose (µmol/L)
2000
1500
1000
500
0
FOR
COL
CGMP
OPN

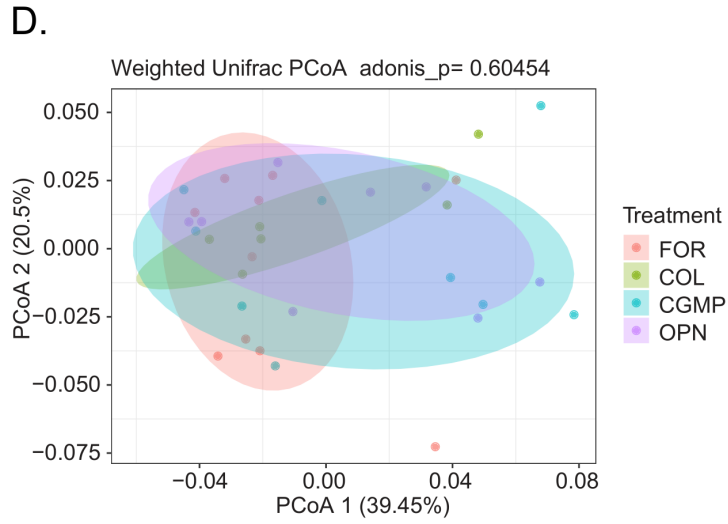

A
Distal IL-1β
IL-1β (ng/g tissue)
P = 0.06
FOR
COL
CGMP
OPN
B
Distal IL-8
IL-8 (ng/g tissue)
P = 0.09
***
C
Distal IL-10
IL-10 (ng/g tissue)
D
Distal I-FABP
I-FABP (ng/g tissue)
E
Distal goblet cells
Goblet cells density (%)
*
F
Colon goblet cells

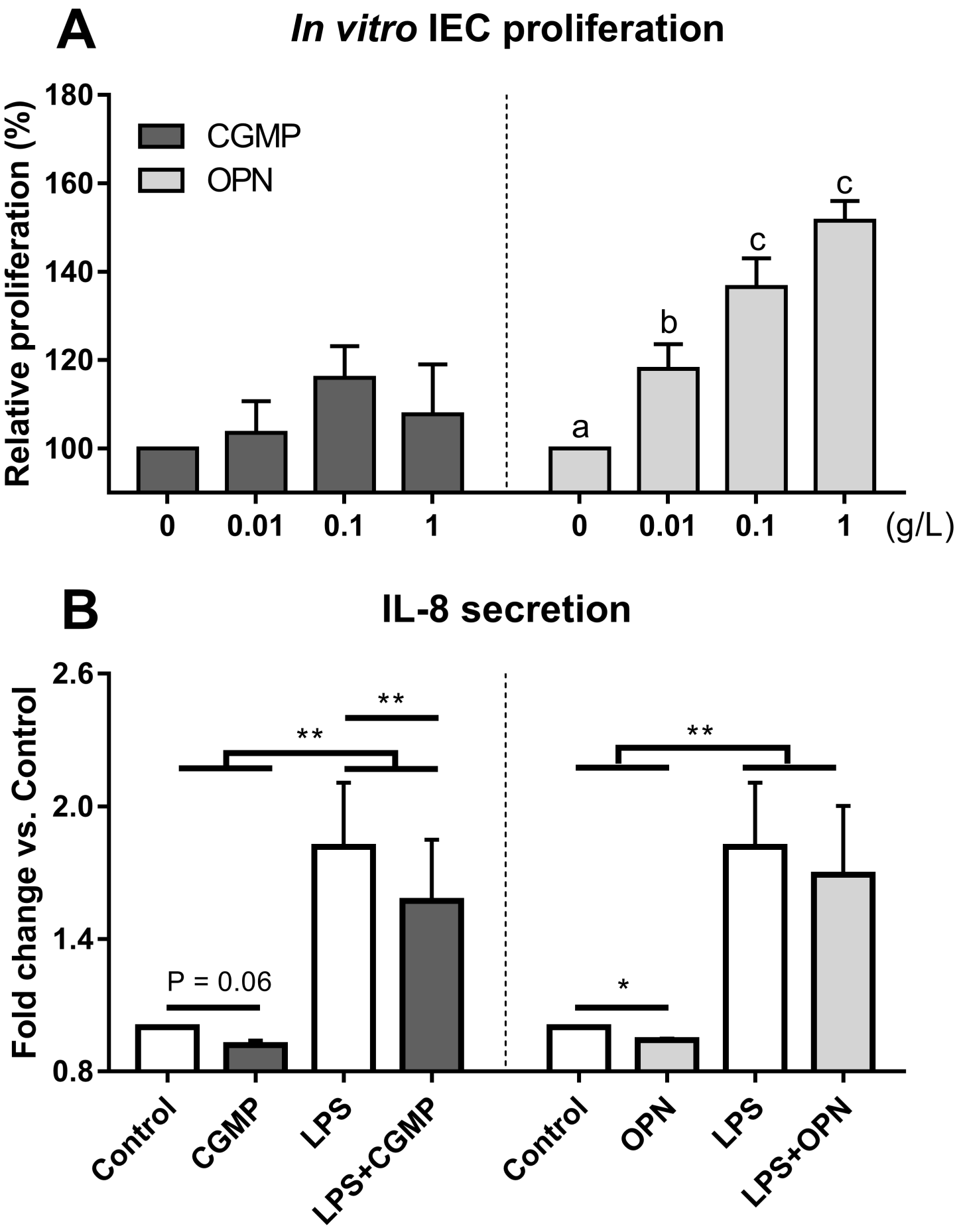

A
Serum glucose
Glucose (mmol/L)
6
5
4
3
2
FOR
COL
CGMP
OPN
B
Plasma IL-1β
IL-1β (pg/mL)
150
100
50
0
FOR
COL
CGMP
OPN
C
Serum iron
Iron (µmmol/L)
8
6
4
2
0
*
FOR
COL
CGMP
OPN
D
Blood help T cells on D3
CD3+CD4+CD8- cells (% lymphcytes)
50
40
30
20
10
0
*
FOR
COL
CGMP
OPN
E
CD4 expression on help T cells on D3
MFI CD4+ (helper T cells)
2500
2000
1500
1000
500
0
P = 0.06
P = 0.07
FOR
COL
CGMP
OPN
F
Neutrophil phagocytic capacity
MFI pHrodo+
10000
8000
6000
5000
0
P = 0.07
FOR
COL
CGMP
OPN

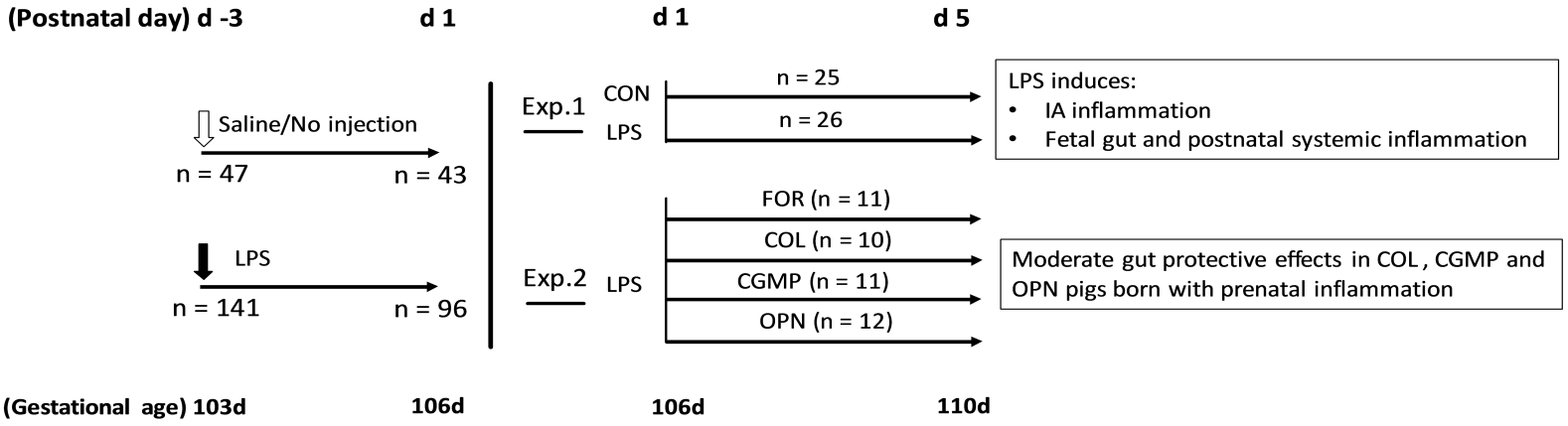

A.
Kruskal−Wallis, p = 0.62
Observed OTU index
300
200
100
FOR
COL
CGMP
OPN
Treatment


B.
Relative abundance (%)
100
75
50
25
0
FOR
COL
CGMP
OPN
Genera
g__unknown_Enterobacteriaceae
others
g__Enterococcus
g__Streptococcus
g__Klebsiella
g__Staphylococcus
g__Clostridium
g__unknown_Clostridiaceae
g__unknown_Leuconostocaceae
g__Lactobacillus
g__unknown_Lactobacillales
g__Pasteurella
g__Streptomyces
g__Weissella
g__unknown_Intrasporangiaceae
g__unknown_Enterococcaceae
g__unknown_Cellulomonadaceae
g__unknown_Micrococcaceae


C.
Unweighted Unifrac PCoA adonis_p= 0.11459
PCoA 2 (11.82%)
0.50
0.25
0.00
−0.25
−0.2
0.0
0.2
0.4
PCoA 1 (19.28%)
Treatment
FOR
COL
CGMP
OPN


D.
Weighted Unifrac PCoA adonis_p= 0.60454
PCoA 2 (20.5%)
0.050
0.025
0.000
−0.025
−0.050
−0.075
−0.04
0.00
0.04
0.08
PCoA 1 (39.45%)
Treatment
FOR
COL
CGMP
OPN


A
In vitro IEC proliferation
Relative proliferation (%)
180
160
140
120
100
CGMP
OPN
a
b
c
c
0
0.01
0.1
1
0
0.01
0.1
1
(g/L)
B
IL-8 secretion
Fold change vs. Control
2.6
2.0
1.4
0.8
**
**
**
P = 0.06
*
Control
CGMP
LPS
LPS+CGMP
Control
OPN
LPS
LPS+OPN


(Postnatal day) d -3
d 1
d 1
d 5
Saline/No injection
n = 47
n = 43
LPS
n = 141
n = 96
Exp.1
CON
LPS
n = 25
n = 26
LPS induces:
• IA inflammation
• Fetal gut and postnatal systemic inflammation
Exp.2
LPS
FOR (n = 11)
COL (n = 10)
CGMP (n = 11)
OPN (n = 12)
Moderate gut protective effects in COL , CGMP and OPN pigs born with prenatal inflammation
(Gestational age) 103d
106d
106d
110d